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Corticotropin-Releasing Hormone Microinfusion in the Central Amygdala Enhances Active Behaviour Responses in the Conditioned Defensive Burying Paradigm

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The central nucleus of the amygdala (CeA) is known to be involved in the regulation of autonomic, neuroendocrine, and behavioural responses in stress situations. The CeA contains large numbers of corticotropin-releasing hormone (CRH) cell bodies, terminals and functional recognition sites. In the present study, the effects of locally infused CRH (30 ng) into the CeA, of freely moving Wistar rats in the conditioned defensive burying paradigm were examined. CRH administered into the CeA increases active (defensive burying and gnawing) behaviour towards a previously electrified probe, which was blocked by pretreatment of α -hCRH, the CRH receptor antagonist. This study clearly shows that the CeA CRH system is involved in the modulation of the CRH-induced behavioural stress response. These findings are discussed in terms of an enhancement of an anxiety response or a shift in coping style balance.

Keywords: behaviour, central amygdaloid nucleus, conditioned shock-probe burying test, CRH, Heart rate, stress

INTRODUCTION

Corticotropin-releasing hormone (CRH), a 41 amino-acid peptide, plays a prominent role in regulating adaptive responses to stress. Considerable evidence suggests that CRH is involved in initiating centrally- and peripherally-mediated endocrine, autonomic and behavioural responses to stressful experiences (Veening *et al.*, 1984; Owens and Nemeroff, 1991).

One of its well known actions is to exert activating effects on the hypothalamic-pituitary-adrenal axis, by releasing adrenocorticotropin, resulting in increases in corticosteroids. When administered intracerebroventricularly (icv) in rats, CRH possesses arousing and anxiogenic properties, which are reflected in autonomic and behavioural activation (Diamant *et al.*, 1991; Diamant *et al.*, 1992; Britton *et al.*, 1982; Koob *et al.*, 1994; Britton, 1990; Koob and Britton, 1990).

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This includes overall increases in sympathetically-mediated responses, such as increases in heart rate and blood pressure, and increases in catecholamine and glucose levels. CRH icv also inhibits cardiac parasympathetic nervous tone (Fisher and Brown, 1991; Fisher, 1991, 1993).

A number of brain sites are currently hypothesized to be particularly important for the behavioural effects evoked by CRH. Especially the nonendocrine CRH system receptors, abundantly prominent in limbic areas, are thought to be involved in the behavioural actions of CRH (Adamec and McKay, 1993; Britton, 1990). Among the limbic regions that contain a majority of CRH-immunoreactive neurons are the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST). The CeA contains the majority of forebrain CRH-immunoreactive neurons that innervate brainstem nuclei such as the parabrachial nucleus, midbrain central grey and dorsal vagal complex (Veening *et al.*, 1984; Danielsen *et al.*, 1989; Gray and Magnuson, 1992; Gray, 1990; Gray, 1993; Bandler and Shipley, 1994). The CeA is thought to participate in the modulation of autonomic and behavioural components of the stress response. Additional data indicate that the CeA is part of the neural circuitry mediating anxiety (Davis *et al.*, 1994). Electrical stimulation of the amygdala in conscious human subjects elicits fully integrated experiences of fear and anxiety (Chapman *et al.*, 1954; Gloor, 1986). In addition, bilateral damage to the human amygdala impairs recognition of particular fear-related, facial expressions (Allman and Brothers, 1994; Adolphs *et al.*, 1994; Tranel and Hyman, 1990). In rats, the CeA is known to mediate fear-related physiological responses, in particular, parasympathetic regulation during both acute and conditioned stress situations (Roozendaal *et al.*, 1990; Kapp *et al.*, 1992; Henke *et al.*, 1991). Moreover, different studies suggest that the CeA is not only involved in the control of parasympathetic output of the autonomic nervous system, but also in the more passive type of behavioural stress response (Roozendaal *et al.*, 1990).

Several studies have provided more direct evidence indicating that the CeA is involved in CRH-mediated stress responses. Immobilization stress induces

increased *c-fos* expression in CeA neurons in the rat. Double staining showed that most of these activated CeA neurons after immobilization stress were CRH containing neurons, although also colocalization with the peptides neurotensin, somatostatin and enkephalin were seen (Honkaniemi *et al.*, 1992; Honkaniemi, 1992). Others found that lesions of the CeA blocked the excitatory effects of CRH in the acoustic startle reflex (Liang *et al.*, 1992). A CRH microdialysis study has demonstrated that CRH is released *in vivo* in the amygdala during immobilization stress (Pich *et al.*, 1995). And recently, an increase in CRH mRNA in the amygdala after restraint stress was found (Kalin *et al.*, 1994). Furthermore, several studies suggest that CRH, when applied locally at the level of cell bodies in the CeA, has an inhibitory influence on CeA output (Rainnie *et al.*, 1992; Wiersma *et al.*, 1993).

The present study was designed to examine the effects of locally applied CRH into the CeA on behavioural and cardiovascular responses in a stress situation in which we expected the CeA to be active, i.e. the conditioned shock-probe burying test (deBoer *et al.*, 1990; Davis, 1992). This paradigm may serve as a behavioural "model" for the study of anxiety and/or anxiolytic agents (Treit, 1985; Craft *et al.* 1988; Korte *et al.* 1992; Tsuda *et al.* 1988). In view of the involvement of the regulation of CeA in the conditioned stress-responses and the known CRH-induced inhibition of CeA output after microinfusion into the CeA in a stress-free condition, we expected a marked effect in behavioural and cardiovascular response of CRH microinfusion into the CeA in this conditioned stress-test.

MATERIALS AND METHODS

Animals

Twenty-seven male Wistar rats, weighing 305–342 g at the beginning of the experiment, were used. The animals were housed individually in perspex cages (25 × 25 × 30 cm) and kept in a temperature controlled room (20 ± 2°C) with a 12-h light-dark cycle (lights on from 08.30 to 20.30). The experiment was performed during the light (between 11.00 and 16.30). Food and

water were available ad libitum except during the experiment (for 2 hrs), when water was not available.

Surgery

For drug infusions, each experimental animal was provided with bilateral permanent stainless-steel brain cannulae (outer diameter 0.3 mm, inner diameter 0.15 mm), aimed just above the central amygdala (coordinates: 6.7 mm rostral to interaural, lateral 4.0 mm to the midline and ventral 6.2 mm below the dura) according to Paxinos and Watson (Paxinos and Watson, 1982). The cannulae were implanted stereotactically and fixed onto the skull by means of stainless-steel screws and dental cement. The outer part of the cannula was protected by a 21-gauge tube except for the last 2 mm at the top. To ensure that the cannulae remained open, a sterile stainless-steel obturator flush was inserted. The cannula and obturator were covered by a polyethylene cap around the protective sleeve. To record the electrocardiogram (ECG), transcutaneous steel electrodes made of standard paper-clips were implanted, one between the scapulae and the other in the middle of the back (Bohus, 1974). The animals were kept under ether anaesthesia during the entire surgical procedure.

Drug Treatment

Synthetic rat/human CRH (CRF; Sigma Chemical Co., St. Louis, MO) and the CRH receptor antagonist, α -helical CRH₉₋₄₁ (α -hCRF; Sigma Chemical Co., St. Louis, MO) were dissolved in artificial cerebrospinal fluid (aCSF) with ascorbic acid (100 μ g/ml aCSF). CRH was administered in a dose of 30 ng/rat per cannula, α -hCRH at dose of 1 μ g/rat per cannula. These doses are based upon results of previous experiments in which CRH and its antagonist showed their agonistic c.q. antagonistic properties (Wiersma *et al.*, 1993; Wiersma *et al.*, 1995). The vehicle was sterile artificial cerebrospinal fluid (aCSF) containing (in mM) 127.64 NaCl, 2.55 KCl, 1.26 CaCl₂ and 0.93 MgCl₂·6H₂O. All compounds were infused in a total volume of 1 μ l in each brain cannula during a 7-min period.

ECG Recording and Analysis

The ECG of freely moving rats was monitored telemetrically by means of a miniature FM transmitter (EDB-ROY, Haren, The Netherlands) as described earlier (Wiersma *et al.*, 1993). The transmitter was connected to the transcutaneous electrodes and secured around the chest by means of a strap. The transmitted signal was received on a commercial FM radio and stored on tape for later, "off-line" analysis. The recorded ECG samples were processed through a cardiometer pulse generator which generated a square wave pulse at each R wave (Olivetti, M24). The time between the onset of two consecutive pulses, the interbeat interval (IBI) was measured within the range of 100 to 220 ms. IBI's were recalculated in beats/min, and expressed as changes in beats per minute (Δ HR).

Behavioural Measurements

The behaviour of the rats during testing in the conditioned shock probe burying test was recorded by means of a keyboard-operated microprocessor (EDB, Haren, The Netherlands). The behaviour was classified into 9 categories: (1) defensive burying = moving toward the shock probe and spraying or pushing the bedding material towards the probe with rapid movements of the snout or forepaws (Pinel and Treit, 1983); (2) gnawing = gnawing on the probe; (3) exploring = investigation of any part of the home cage; (4) rearing = standing on the hind-legs only; forepaws either free or leaning against the wall of the cage; (5) sniffing = sniffing in the air with paws on the floor; (6) grooming = wiping the fur with forepaws and tongue (washing); (7) eating = chewing chow or faeces; (8) immobility = the animal is completely motionless and the body weight is supported by its limbs; (9) resting/sleeping = inactive with eyes open or closed. Since burying and gnawing are both expressions of an active behavioural response, these two behaviours were taken together to form "active behaviour".

The duration and the frequency of the elements were recorded and expressed as the percentage of the total observation period.

Procedure

After at least ten days recovery from surgery, the rats were trained and tested. For at least a few hours during the three days before the start of the experiments, rats were habituated to the strap holding the transmitter, and to the infusion procedure. Rats were subjected to the conditioned shock-probe defensive burying (CDB) test. The test was performed in each individual's home cage to avoid nonspecific responses of the neuroendocrine and cardiovascular systems by intercage transfer and handling (deBoer *et al.*, 1990; Korte *et al.*, 1992). The floor was covered with wood shavings. A removable teflon probe (6.5 cm long, 1 cm in diameter) was placed 6 cm above the floor, inserted through a small hole in the centre of the front wall of the Plexiglas cage. Electric current (1.5 mA) was administered through two exposed wires (0.5 mm in diameter) each wrapped (25 times) independently around the probe. On day 1, the shock probe was inserted in the home cage for 15 min. Upon touching the probe, the rat received an electric shock of 1.5 mA/contact. The shock circuit was left on during the entire period (repeated shock-probe procedure as described by Treit and Fundytus, 1988). All rats usually received one brief electric shock. Twenty-four hours later, on the test day, the procedure was identical except that no electric current was applied to the probe and behavioural and cardiovascular responses were measured. Thus, the procedure investigated the conditioned consequence of former punishment rather than the direct effect of the shock. It is known that rats can cope with a well-localized source of aversive stimulation within their home cages by means of an active (i.e. burying and gnawing) or a passive (i.e. freezing) form of avoidance responding (deBoer *et al.*, 1990). On this test day, microinfusion into the CeA was applied. Each animal was pretreated either with α -hCRH or vehicle, 50 minutes before the CRH or aCSF infusion. The pretreatment was given to the experimental animal by the experimenter, after which each rat was connected to the infusion tubes filled with the second drug infusion solution. Immediately after the pretreatment each rat was placed into its home-cage, connected with ECG transmitter and infusion tubes, filled with

the second drug infusion solution. At $t = 0$ either CRH or aCSF was microinfused in the undisturbed, freely moving rat for 7 min. At $t = 10$ min the non-electrified probe was inserted in the home cage. Heart rate and behaviour were measured for 1 min periods before infusion (at $t = -1$), just after infusion but before probe presentation (at $t = 7$ and $t = 9$), during presentation of the nonelectrified probe (at $t = 12, 15, 20$ and 25) and after removal of the probe (at $t = 35, 45$ and 55 min). Basal measurement was taken at $t = -1$ min.

Histology

At the end of the experiment, the rats were deeply anaesthetized with sodium pentobarbital (90 mg/kg ip) and perfused intracardially with saline followed by a 4% formaldehyde solution. The brains were postfixed in the same fixative for at least one week. Frozen sections of 40 μ m were cut and the tip of the cannula placement was determined on stained (Cresyl violet staining) sections.

Statistics

The results are presented as means \pm SEM. Heart rate and behavioural data were evaluated using an analysis of variance with repeated measures (ANOVA) followed by the Mann-Whitney-U-test. A probability level of $p < 0.05$ was taken as the criterion for significance.

RESULTS

Histology

Histological examination revealed that three of the animals had to be excluded from further analysis because of improper bilateral cannulae placements. The cannulae tips had to be localized just above or entering the dorsal edge of the CeA.

Cardiac Responses

Figure 1 shows the changes in mean heart rate, before, during and after microinfusion and the presentation of

the non-electrified probe in the home cage of the rats on the test day, compared to the basal sample ($t = -1$). All three groups showed an increase in mean heart rate starting just after the microinfusion, reaching a maximum of about 40 beats/min. Upon presentation of the non-electrified probe, an abrupt increase in heart rate occurred, immediately after which it slowly decreased and returned to basal levels at the last time sample. No significant differences were found between the three treatment groups by analysis of variance. Also baseline heart rate levels showed no significant differences: CSF/CSF 416.0 sem 8.02, CSF/CRH 421.9 sem 11.63, α -hCRH/CRH 430.1 sem 15.1.

Behavioural Responses

Figure 2 shows the behavioural responses of the rats in the three different infusion treatments during the test session. The main behavioural responses the rats displayed were burying and immobility. Some rats showed gnawing responses on the probe. The gnawing response was only seen in the rats receiving CRH only. The active behavioural response (burying and gnawing) was clearly affected by the different treat-

ments (Fig. 2a). Figure 2a shows that during the first 2 min of the presentation of the non-electrified probe, all the treatment-groups showed an increase of active behaviour. This was followed by quick decrease of this behaviour in both the vehicle and the α -hCRH + CRH group. It returned to basal levels at $t = 15$ min. The CRH-treated animals showed a well pronounced increase in active behaviour, which lasted during the entire time when the non-electrified probe was presented. The active behavioural response returned to baseline after the probe was removed. Comparison of vehicle and CRH treatment groups with the repeated measures ANOVA revealed a significant main effect of treatment ($F_{1,13} = 10.254$, $P < 0.01$) and an interaction between treatment and time ($F_{4,52} = 4.889$, $P < 0.005$). Analysis of the separate sample times revealed significant differences in active behavioural responses at $t = 15$ ($P < 0.01$) and $t = 20$ min ($P < 0.05$) between the CSF and the CRH group. The active behavioural response was blocked by pretreatment of α -hCRH. In comparison of the α -hCRH + CRH treated group with the CRH group, the ANOVA showed a significant treatment effect ($F_{1,10} = 6.413$, $P < 0.05$) and a significant interaction between treatment and time ($F_{4,40} =$

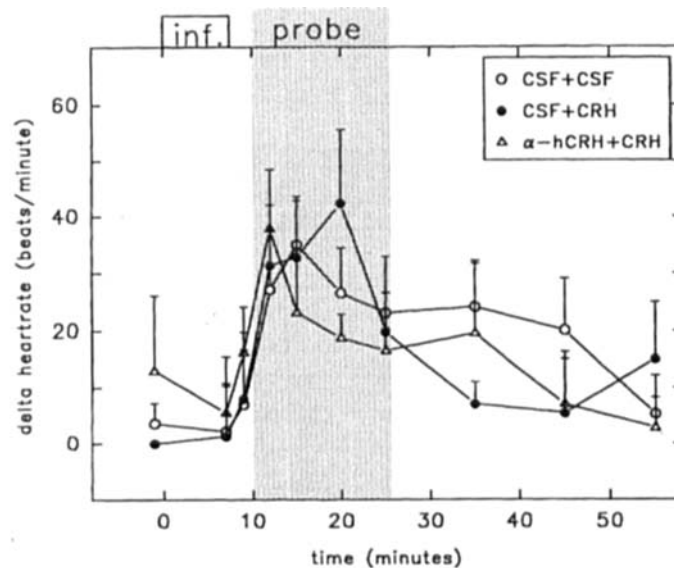


FIGURE 1 Changes in heart rate (\pm S.E.M.) as a consequence of infusion of CRH (30 ng in 1 μ l) or artificial-CSF (1 μ l) for 7 min at $t = 0$ min into the CeA and pretreated with aCSF (1 μ l) or α -hCRH (1 μ g in 1 μ l) at $t = 50$ min in the conditioned shock-probe burying test. The re-exposure (24 hrs after the shock-session) to the non-electrified probe was presented in the homecage at $t = 10$ min till $t = 25$ min. CRH-group, $n = 8$ (●); vehicle-group, $n = 9$ (○); α -hCRH + CRH group, $n = 5$ (△).

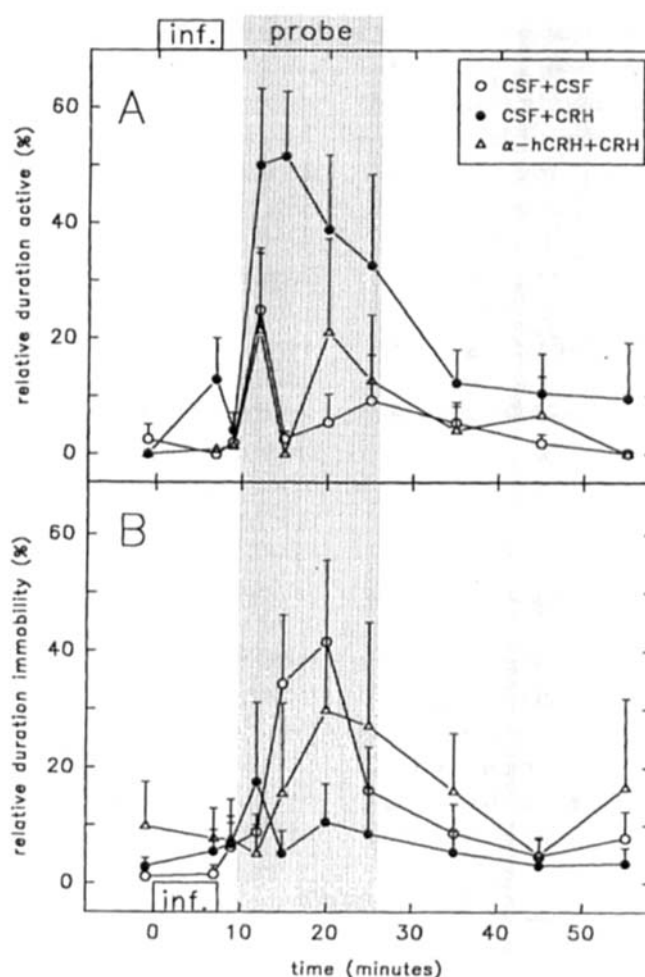


FIGURE 2 Percentage of time spent in various behaviours as a consequence of infusion of CRH (30 ng in 1 μ l) or artificial-CSF (1 μ l) for 7 min at $t = 0$ min into the CeA and pretreated with aCSF (1 μ l) or α -hCRH (1 μ g in 1 μ l) at $t = 50$ min in the conditioned shock-probe burying test. The re-exposure (24 hrs after the shock-session) to the non-electrified probe was presented in the homecage at $t = 10$ min till $t = 25$ min. Active behaviour (burying behaviour and gnawing) (2A) and immobile behaviour (2B) are shown. CRH-group, $n = 7$ (●); vehicle-group, $n = 9$ (○) α -hCRH + CRH group, $n = 5$ (△).

3.427, $P < 0.05$). The Mann-Whitney U Test indicated that only at $t = 15$, a significant reduction in the α -hCRH pretreated group was found ($P < 0.05$). Analysis of variance did reveal neither a significant treatment effect nor treatment X time interaction between the vehicle and the α -hCRH + CRH group.

Immobility (Fig. 2b) showed the opposite effect compared to the active behavioural response. After the probe was presented, rats showed little immobile behaviour but after 5 minutes ($t = 15$) an increase was observed in the vehicle group. Also the α -hCRH + CRH group showed some increase in immobility,

starting at $t = 15$, but this was not as pronounced as the vehicle-group. However the CRH-treated animals did not show any increase in immobile behaviour during the entire procedure. Analysis of variance revealed a significant interaction between treatment and time ($F_{4,52} = 2.693$, $P < 0.05$) only in the vehicle and the CRH-treated rats. Analysis of the separate time samples showed a significant effect at $t = 15$ ($P < 0.05$). No significant differences were found in immobility behaviour between the vehicle and α -hCRH + CRH treatment, or between CRH treated rats and α -hCRH + CRH treated rats.

The occurrence of the other behaviours, i.e. grooming, exploring, rearing, and resting/sleeping, did not differ significantly between the different treatments ($P > 0.05$).

DISCUSSION

CRH microinfusion into the CeA in a conditioned stress paradigm resulted in a significant increase in "active" behaviour, which includes defensive burying and gnawing behaviour and a decrease in immobility behaviour. The cardiovascular response was not affected by the locally applied CRH. Pretreatment with the CRH-receptor antagonist resulted in a significant blockade of the CRH-induced behavioural changes.

These results demonstrate that the CRH system in the CeA is involved in the modulation of behaviour during stressful conditions. Earlier work done in our laboratory in stress-free conditions demonstrated that CRH microinfused into the CeA resulted in tachycardia and behavioural activation without an activation of plasma adrenaline, noradrenaline and corticosterone. Therefore it is likely that the CRH-induced effects after microinfusion in the CeA, both in stress-free and conditioned stress situations (this study) are due to modulation of the non-endocrine CRH system in the CeA itself (Wiersma *et al.*, 1993). As CRH (30ng) microinfusion in a stress-free condition into the basolateral nucleus of the amygdala did not result in any significances of behavioural, cardiovascular or endocrine activation at all (Wiersma *et al.*, 1996), the present results are unlikely to be due to spread of CRH beyond the CeA.

In contrast to our observations in stress-free conditions (Wiersma *et al.*, 1993), the results of the present study show a clear dissociation between the behavioural and heart rate responses. The absence of any effects on heart rate after CRH infusion in the CeA in the conditioned stress situation can be explained by the fact that the stressor is known to induce a strong activation of sympathetic output (deBoer *et al.*, 1990). The expected parasympathetic inhibition after infusion of CRH in the CeA may therefore be masked by the probable near maximal sympathetic activation.

The CRH-induced increase in active behaviour is in accordance with a study which infused CRH icv (Diamant *et al.* 1992). As a similar dissociation was found between conditioned behaviour and heart rate responses, we suggest that the effects found by Diamant and colleagues (Diamant *et al.*, 1992) after icv CRH can be a result of CRH-induced modulation of CeA output. This indicates that the site of action to induce behavioural effects evoked by CRH icv may well be within the CeA-CRH system.

The finding that the CRH receptor antagonist suppresses CRH-induced increases in active behaviour is consistent with other studies showing that the CRH antagonist icv reverses stress-induced behavioural responses (Heinrichs *et al.*, 1994; Korte *et al.*, 1994; Adamec and McKay, 1993). A role for CRH receptors in behavioural stress responses has been shown before in the CeA (Heinrichs *et al.*, 1992; Rassnick *et al.*, 1993; Swiergiel *et al.*, 1993).

Earlier studies interpreted the effects of CRH applied locally in the CeA as inhibitory to CeA output (Wiersma *et al.*, 1993). The increase in active behavioural responses and decrease in immobility responses seen in this study could be a result of an inhibition of CeA output as well. Such a CRH-mediated inhibition in the CeA neurons may be due to an autoinhibition by somatodendritic CRH autoreceptors, which is supported by electrophysiological studies as well (Rainie *et al.*, 1992). Despite the fact that the behavioural effects of CRH in the CeA are most likely mediated by CRH receptors, none of the CRH receptors described this far (CRH-1 receptor and 2α and 2β receptors) are abundantly present in the CeA (Potter *et al.*, 1994; Perrin *et al.*, 1995; Lovenberg *et al.*, 1995). The explanation of the present results in terms of CRH receptor-mediated processes will have to wait for a further molecular analysis of CRH signal transduction.

An increase in burying behaviour in the conditioned shock probe burying test is generally considered to be an expression of an increased level of anxiety (Pinel and Treit, 1983; Treit, 1985). From that point of view, the present results indicate that the CRH system in the CeA is involved in anxiety, resulting in an increase in anxiety levels. However, at the same time, we observed an inhibition in immobility behaviour,

known as being an expression of anxiety levels in different test as well (Roozendaal *et al.*, 1990, 1991). A decrease in immobility responses in this test suggest a decrease in anxiety levels. This is contradictory to the conclusion of an increase level of anxiety as a result of the increased levels of burying behaviour in the same test. Accordingly the inhibition in immobility behaviour cannot be ascribed to a mutual exclusion of burying/gnawing and immobility behaviour.

In view of recent studies on stress and coping, an alternative interpretation can be given. It is known that rats display different behavioural response styles when coping with adverse environmental events (Benus *et al.*, 1991; Bohus *et al.*, 1987). One type of response is characterized by immobility and suppression of environmentally-directed activities, i.e., the passive-conservation-withdrawal mode of response. The other response style is characterized by active responding whereby an animal displays much locomotor activity in attempting to escape from or to deal with an external threat i.e. the flight/fight defense pattern of reaction. These coping styles differ not only at the level of behaviour (Benus *et al.*, 1991), but also in physiology (Koolhaas, 1994; Bohus *et al.*, 1987) and chemical neuroanatomy (Compaan *et al.*, 1993). Hence, this CRH system in the CeA may play a role in selecting the appropriate behavioural coping style in response to environmental challenges (Korte *et al.*, 1994). This interpretation is supported by peptidergic manipulations of the CeA in normal male rats (Roozendaal *et al.*, 1992a) and in two strains of rats known to adopt an active versus a passive coping style, respectively (Roozendaal *et al.*, 1992b). Thus, the CRH-induced increase in active behaviour after infusion into the CeA, could be a result of a shift in the balance of the different coping styles towards a more active coping style. This suggests that in Wistar rats a shift in the balance between the active and passive coping styles may occur. Accordingly it can be concluded that a strict separation between the two coping styles is not present in Wistar rats after manipulation of the CeA with peptidergic microinfusions. However, an interpretation in terms of enhanced anxiety cannot be ruled out.

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